AD

ENZYMATIC HYDROLYSIS OF CELLULOSIC MATERIALS TO FERMENTABLE SUGARS FOR THE PRODUCTION OF ETHANOL

PROGRESS REPORT

TO

U. S. DEPARTMENT OF ENERGY

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By

Environmental Sciences & Engineering Division
Food Sciences Laboratory

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UNITED STATES ARMY
NATICK RESEARCH and DEVELOPMENT COMMAND
NATICK, MASSACHUSETTS 01760

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Spano, Leo A.

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U.S. Army Natick Research, Development and Engineering Center
ATTN: RDNS-WSC-C
Kansas St., Natick, MA 01760-5020

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14. ABSTRACT

This report summarizes the work performed during the quarter ended September 30, 1980 by U.S. Army Research and Development Command Environmental Services and Engineering Division Foot Services Laboratory under an interagency agreement between the U.S. Army Research and Development Laboratories and the U.S. Department of Energy to conduct basic and applied research in the conversion of cellulose to glucose sugar through enzymatic hydrolysis for the production of ethanol. Included are the financial expenditures incurred during quarter and the work planned for the next quarter.

15. SUBJECT TERMS

CELLULOSE	WASTES	FERMENTATION
GLUCOSE	FUNGI	TRICHODERMA REESEI
ENZYMES	FUELS	ETHANOL
HYDROLYSIS	ENERGY	

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QUARTERLY HIGHLIGHTS

TASK I

1. The composition and properties of the cellulase enzyme complex from Trichoderma reesei strains presently available are similar regardless of the strain or growth substrates although higher levels of enzyme are secreted by the enhanced cellulase mutants.

2. Endo- and exo-glucanases are secreted simultaneously during the fermentation with exo-glucanase accounting for approximately 68% of the total protein.

3. All Trichoderma cellulase preparations have specific enzyme activities of about 0.6 filter paper cellulase units per mg of protein and show saccharification efficiencies of 15-26% in 16 hour hydrolysis of 15% substrate.

TASK II

1. Poplar shavings were compression mill pretreated most effectively at an initial moisture content of 12%. With only 15 passes through the rolls of our 10" x 20" mill, this feedstock at 12% initial moisture content was ten times more susceptible to T. reesei cellulase than the untreated control. A 4-fold reduction in the number of passes compared to air dry shavings was noted.

2. Further reduction in the number of passes have been achieved by increasing the pressure on the feedstock passing between the mill rolls. A 4-high

metals cold rolling mill was used on newspaper with good hydrolysis results obtained in six passes or less. Energy consumption was approximately 0.21 kwh/lb.

TASK III

1. The specific growth rate of Aspergillus phoenicis QM329 is 0.4 hr^{-1} at 35° and pH 4.5.

TASK IV

1. Shake flask hydrolysis runs using NEP200 and HMNP with glass beads can be successfully scaled up in an attritor mill. Power costs will determine the feasibility of using such a process.

2. The MCG77 strain of Trichoderma reesei produced more sugar on NEP200 than both QM9414 and C30 at three different enzyme strengths, with and without glass beads.

3. Unshaken flasks using C30 enzyme and NEP200 produce nearly as much sugar as shaken flasks. Thus a reduction in power cost for agitation can be realized.

INTRODUCTION

On 26 February 1976, the U. S. Army R&D Laboratories signed an Interagency Agreement with the U. S. Department of Energy (formerly U. S. Energy Research and Development Administration) to provide the necessary personnel, facilities, services, materials and documentation to conduct basic and applied research in the conversion of cellulose to glucose sugar by means of enzymatic hydrolysis including:

a. Continuation and expansion of mutants' work to develop fungus strains that produce more cellulase or otherwise enhance the conversion process to include development of a rapid plate assay to enhance capability to screen cultures.

b. Investigate and optimize all variables that control enzyme production including nutrients, temperature, pressure, acidity, cellulose slurry concentration, fungus growth, dissolved oxygen, antifoam concentration, biochemistry and kinetics of the process, etc.

c. Investigate and study cellulase preparations from other active cellulolytic organisms including thermophiles; adsorption and desorption of cellulase on cellulose; organisms to degrade or convert lignin wastes, and the syrups produced from various cellulose substrates; investigate the use of biocides, if necessary.

d. Investigate and optimize the conditions for operation of hydrolysis reactors to include concentration and feed rate of cellulose slurry, enzyme concentration, pretreatment of cellulose substrates, glucose syrup concentration, temperature, acidity, residence time, recovery of enzymes, fungi, glucose process control, etc.

e. Economic studies of the process considering cellulosic waste preparation, sugar production rates, recovery and/or waste of enzymes, recovery, concentration and purification of sugars, solids and other by-products. Studies of economic scale of operations.

f. Study of the utilization of glucose syrups, solid residue (lignin) and fungus grown during enzyme production particularly for direct or indirect conversion to energy forms.

Major areas of work emphasized during the past reporting period were:

I Applied Research and Process Development.

II Substrate and Substrate Pretreatment

III Enzyme Production (Prepilot Scale)

IV Saccharification (Prepilot Scale)

The objective of this progress report is to discuss what has been accomplished during this past quarter and what is being planned for next quarter.

Included also are the financial expenditures incurred during this performance period.

TASK I APPLIED RESEARCH AND PROCESS DEVELOPMENT

OBJECTIVES

The objectives of the Enzyme Technology program are to carry out laboratory research on cellulase production and saccharification of waste cellulose by cellulase that will lay the foundation and define the parameters for a practical process. Our approach is to (a) investigate the physiological, biochemical and genetic factors involved in induction, synthesis, and secretion of the enzyme; (b) utilize this information to maximize cellulase yields and reduce the costs of enzyme production; (c) to study the interactions of the cellulase enzymes with their substrates including the effects of levels of the various enzyme components, effects of inhibitors including the products of the reaction, and the effects of cellulose structure, degree of crystallinity, and admixture with impurities such as lignin on the rate and extent of the hydrolysis reaction; and (d) to utilize this information to maximize sugar yields and reduce the cost of the saccharification process.

ACCOMPLISHMENTS

Effects of Strain and Substrate on Composition, Properties, and Saccharification Potential of Trichoderma reesei Cellulase (Reference 1)

Much recent effort at Natick and elsewhere has been directed to reduction of the high cost of Trichoderma cellulase by improvements in fermentation conditions and by development of new mutant strains. Studies reported last quarter (Reference 2) show that all presently available strains are induced

for cellulase by cellulose, lactose, and sophorose, and all are repressed by glucose. Cellulase yields of the mutants as compared to the wild strain are enhanced about 6 fold on 6% lactose, about 20 fold on 6% ball milled cellulose, and about 3 fold on 6% roll milled cotton (Table 1). Despite this variation in yields, the specific activities of the cellulases remained fairly constant, 0.43-0.46 cellulase units per mg of protein when grown on lactose and 0.6-0.7 cellulase units per mg of protein when grown on cellulose (Table 2). Furthermore, analysis of the cellulases by HPLC (Reference 3) showed only slight variations in protein profiles either for the different strains grown under identical conditions (Figure 1) or a single strain grown on different substrates (Figure 2). In all cases exo-β-glucanase components accounted for 56-72% and endo-β-glucanase components for 28-44% of the total protein (Table 3). Protein profiles did change as the culture aged (Figure 3). In the example shown (Figure 3) at all times about 68% of the total protein was exo-β-glucanase, but with time the portion of this in the fast moving peak (zone 2) increased at the expense of the slow moving peak (zone 4).

Enzymes produced by the different mutants were evaluated for saccharification potential at different activity levels using ball milled cellulose (BW200), ball milled newspaper (NEP200), microcrystalline cellulose (Avicel) and absorbent cotton (Table 4, Figure 4). With low levels of enzyme activity (0.5 FPU/ml) and short reaction times (four hours) enzymes from all the mutants gave approximately the same amount of sugar from BW200, NEP200, or Avicel. At longer times or higher activity levels the MCG77 enzyme was more effective than the QM9414 enzyme or the C30 enzyme. In 24-hour hydrolyses on BW200, Avicel, and cotton QM9414 enzyme gave more sugar than the C30 enzyme. On NEP200 C30 enzyme produced more sugar at all activity levels than QM9414 enzyme, and

at low levels produced slightly more than MCG77 enzyme.

The effect of time on the extent of hydrolysis was also evaluated. It was found that with BW200, NEP200, and Avicel all enzymes gave twice as much sugar at 24 hours as at 4 hours. With cotton there was a 3-fold increase during this same time period. An additional 24 hours resulted in a 30-50% increase with Avicel and cotton, but only a 10% increase with BW200 and NEP200. During saccharification the predominent sugar is glucose. Table 5 lists the sugars found in digests by QM9414 enzyme at 24 hours for the four substrates. Similar patterns were obtained by enzymes from other mutants. Enzyme utilization efficiency (percent of predicted saccharification based on enzyme units) on 15% substrate at 16 hours was approximately 15% for Avicel, 24% for BW200, and 26% for NEP200 for all enzyme preparations (Table 6).

PLANS, NEXT QUARTER

Work will continue on induction studies, optimization of enzyme production in batch culture on newspaper and in continuous culture on pure cellulose, on enzyme stabilization, and on enzyme substrate interactions.

REFERENCES

1. Bissett, F. H.; Andreotti, R. E.; and Mandels, M. 1980. Effects of Strain and Substrate on Composition, Properties, and Saccharification Potential of Trichoderma reesei Cellulase. Proceedings Second International Symposium on Bioconversion and Biochemical Engineering. New Delhi, India. March 1980.

2. Andreotti, R. E.; Medeiros, J. E.; Roche, C.; and Mandels, M. 1980. Effects of Strain and Substrate on Production of Cellulases by Trichoderma reesei Mutants. Proceedings Second International Symposium on Bioconversion and Biochemical Engineering. New Delhi, India. March 1980.

3. Bissett, F. H. 1979. Analysis of Cellulase Proteins by High-Performance Liquid Chromatography. J. Chromat. 178: 515-523.

Table 1. Effect of Strain and Substrate on Cellulase Production

Strain	6% Substrate	Soluble Protein mg/ml	Filter Paper Activity u/ml	Productivity u/l hr
QM6a	FB Cotton	7.4	5.0	15
	BW200	*0.8*	*0.6*	*1.8*
QM9414	FB Cotton	13.0	8.4	25
	BW200	*8.7*	*5.0*	*30*
MCG77	FB Cotton	16.2	10.7	32
	BW200	*9.6*	*7.4*	*45*
Rut C30	FB Cotton	20.6	13.6	41
	BW200	*17.8*	*11.9*	*73*

BW200 = Ball milled pulp -200 mesh
FB Cotton = Absorbent cotton processed for one minute (10 mil gap) on Farrel Birmingham 2 roll mill (13)
Cultures grown in fermentors at 28°C with pH controlled not to go below 3.0
Enzyme units = micromoles glucose produced per minute in standard assay (6)

(Bissett et al. 1980)

Table 2. Effect of Strain and Substrate on Specific Activity

Strain	6% Substrate	Specific Activity-Reducing Sugar u/mg CMC	FP	Avicel	BW200	Specific Fluidity ΔF/dt mg
QM6a	FB Cotton	12.6	0.7	0.47	0.63	21
QM9414	FB Cotton	7.8	0.7	0.46	0.56	34.5
	BW200	*11*	*0.6*	*0.44*	*0.61*	*31.2*
MCG77	FB Cotton	6.5	0.7	0.4	0.65	27.3
	BW200	*15*	*0.9*	*0.4*	*0.58*	*33*
	Lactose	9.7	0.46	0.3	0.46	22.8
C30	FB Cotton	7.9	0.74	0.45	0.6	24
	BW200	*11*	*0.64*	*0.45*	*0.52*	*31.2*

BW200 = Ball milled pulp -200 mesh
FB Cotton = Absorbent cotton processed for one minute (10 mil gap) on Farrel Birmingham 2 roll mill (13)
Cultures grown in fermentors at 28°C with pH controlled not to go below 3.0
Enzyme units = micromoles glucose produced per minute in standard assay (6)

(Bissett et al. 1980)

Table 3. Percentage of Exo and Endo-1,4-β-Glucanase in *T. reesei* Strains

Strain	Growth Substrate	% Endo	% Exo A*	% Exo B*	Total % Exo
QM6a	Cotton	34	16	50	66
QM9414	Cotton	34	17.5	49	66.5
	BW200	*37*	*17*	*46*	*63*
MCG77	Cotton	39	12	49	61
	BW200	*37*	*20*	*43*	*63*
	Lactose	44	10	46	56
C30	Cotton	28	13	59	72
	BW200	*31*	*19*	*50*	*69*

*Exo A = Zone 2 (fig 1) Exo B = Zone 4

(Bissett et al. 1980)

Table 4. Sugar Produced at Different Enzyme Levels

Substrate	Enzyme FPU/ml	Reducing Sugar mg/ml			% Conversion		
		9414	MCG77	C30	9414	MCG77	C30
15% BW200	0.5	35	35	33	18	18	17
	1.0	55	52	42	29	27	22
	2.0	68	73	56	36	39	30
	4.0	--	96	70		52	37
15% Avicel	0.5	27	26	21	14	13	11
	1.0	33	36	28	17	19	15
	2.0	40	47	37	21	25	19
	4.0	--	60	45		32	24
15% NEP200	0.5	30	32	35	25*	26*	27*
	1.0	38	48	44	30	38	35
	2.0	48	63	54	38	50	43
	4.0	--	78	61		63	49
5% Cotton	0.5	6.5	9.5	4	11	16.5	7
	1.0	8.5	11	5	15	19	9
	2.0	10	13	6.5	17	23	11
	4.0	--	16.5	8		29	14

All enzymes grown on 6% BW200

Hydrolysis conditions: pH 4.8, 50°C, 24 hrs.

* % conversion of NEP200 - based on 10.5% cellulose as approximately 30% of NEP200 is not cellulose

(Bissett et al. 1980)

Table 5. Saccharification of Cellulose by QM9414

Substrate	Xylose	Glucose	U_1	U_2	Cellobiose	Gentiobiose	Trimer	Total	% Hydrolysis
15% BW200	3.3	70.2	0.8	1.5	13.3	4.0	1.0	94.0	51
15% Avicel	*0.6*	*33.6*		*0.6*	*5.7*	*3.0*	*0.9*	*44.*	*23*
15% NEP200	5.9	54.8		0.6	5.7	0.5		67.5	54
5% Cotton		*11.6*			*1.8*			*13.4*	*23*

Hydrolysis conditions: pH 4.8, 50^0, 24 hr.

Cellulase activity = 0.027 FPU/mg substrate

Sugar concentrations are in mg/ml as determined by L.C.

(Bissett et al. 1980)

Table 6. Enzyme Utilization Efficiency

Substrate	Act. FPU/ml	% Enzyme Utilization		
		9414	MCG77	C30
15% BW200	0.5	21	22	19
	1.0	*25*	*25*	*22*
	2.0	26	27	22
	4.0		*27*	*21*
	Ave	24	25	21
15% Avicel	0.5	17	16	12
	1.0	*15*	*18*	*11*
	2.0	13	17	12
	4.0		*16*	*13*
	Ave	15	17	12
15% NEP200	0.5	23	26	28
	1.0	*23*	*32*	*27*
	2.0	27	33	30
	4.0	--	--	--
	Ave	24	30	28

(Bissett et al. 1980)

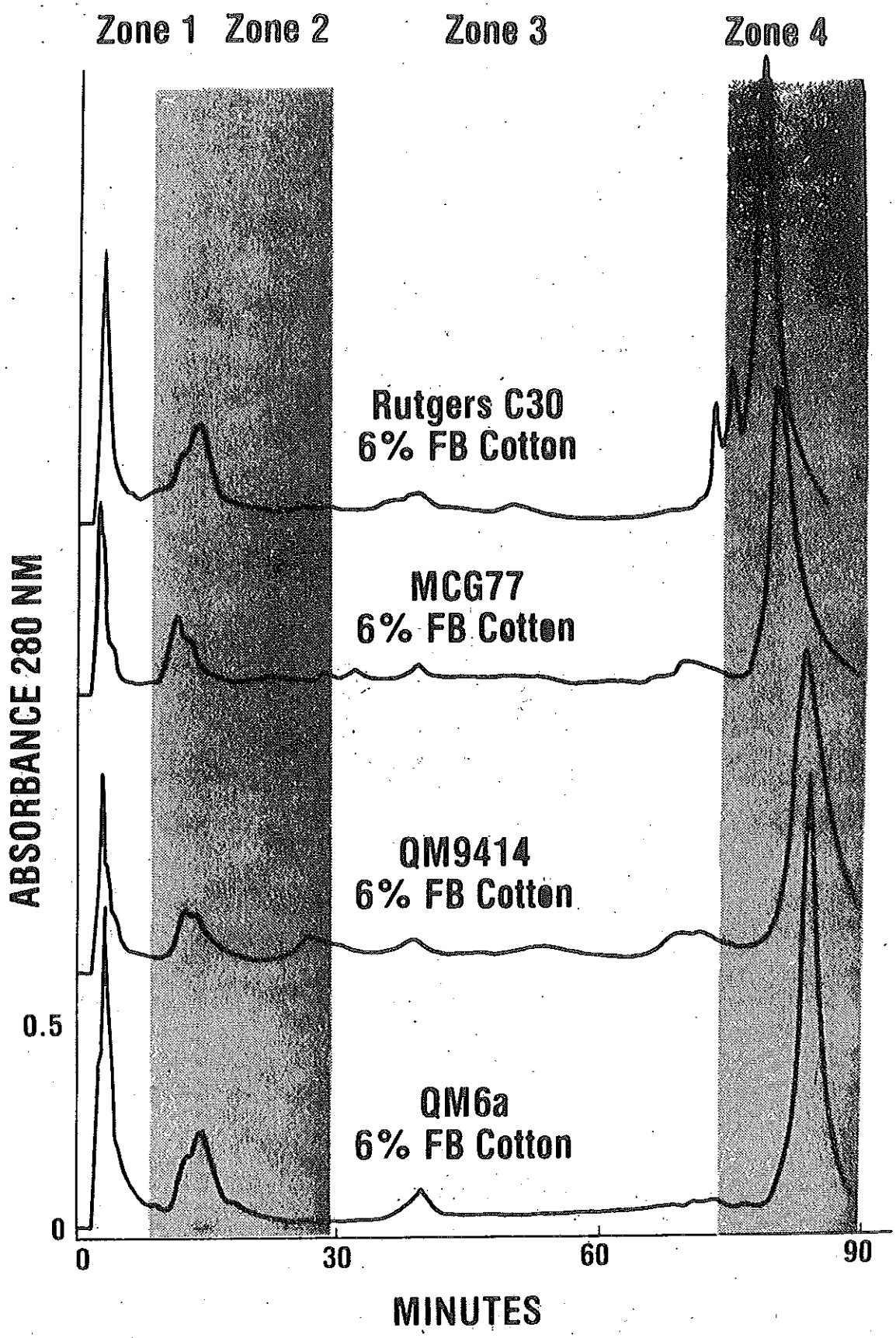

Figure 1. HPLC protein profiles of Trichoderma reesei strains.

Solvent: 20 mM NaH_2PO_4-3mM NaN_3, pH 6.2.

Components eluted with a NaCl salt gradient.

(Bissett et al. 1980)

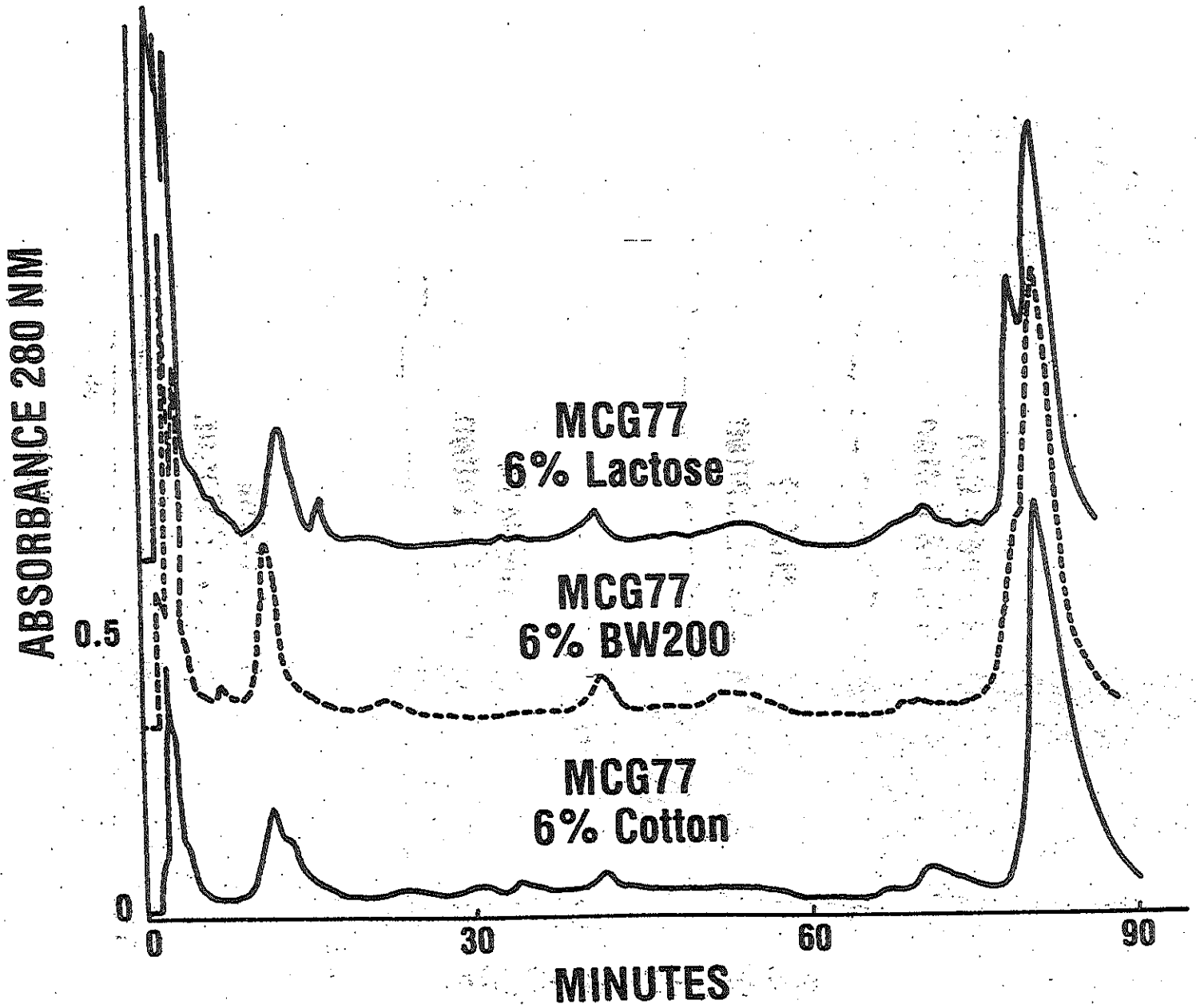

Figure 2. HPLC protein profiles of enzyme produced by MCG77.

(Bissett et al. 1980)

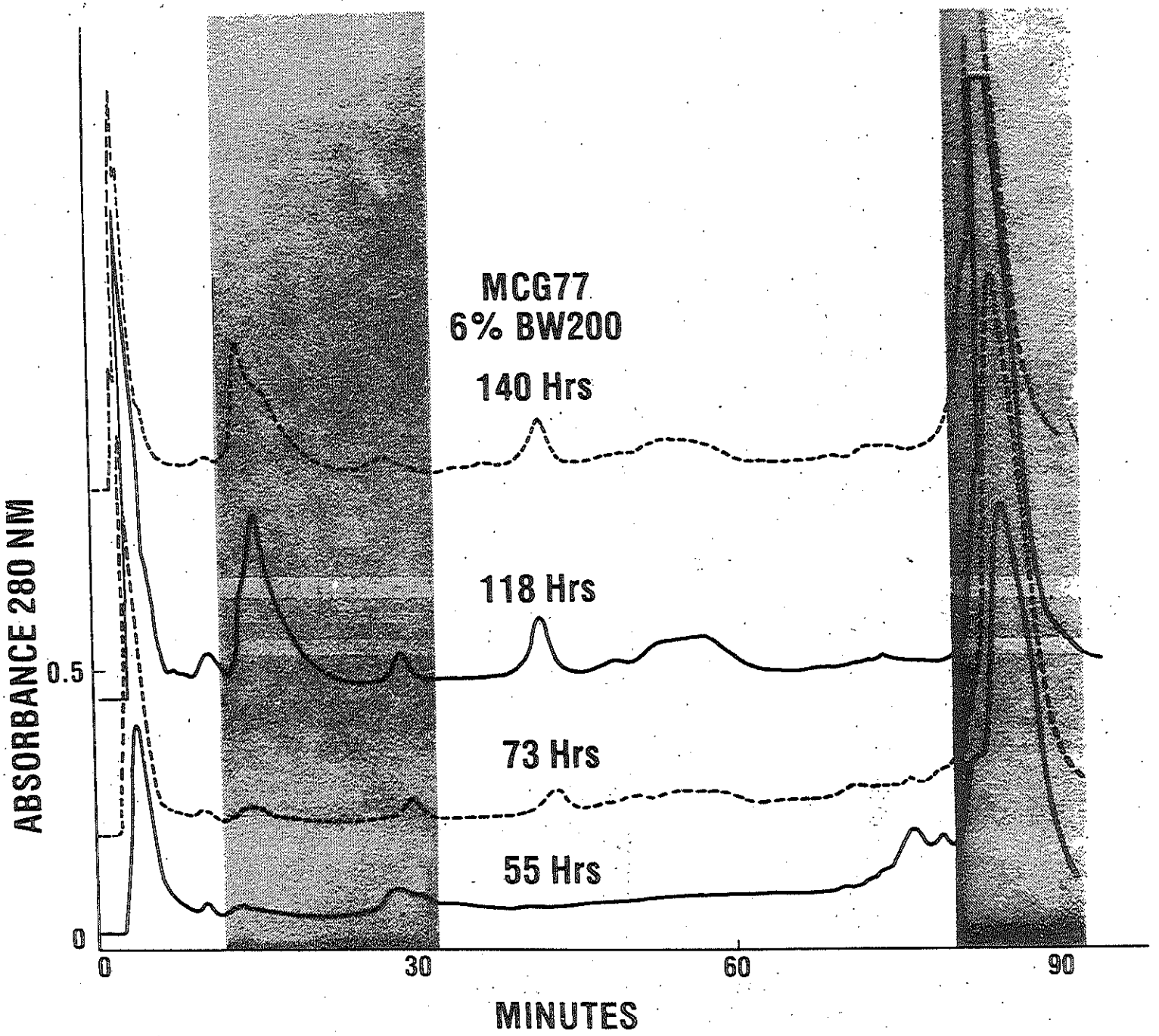

Figure 3. Enzyme production during fermentation.

(Bissett et al. 1980)

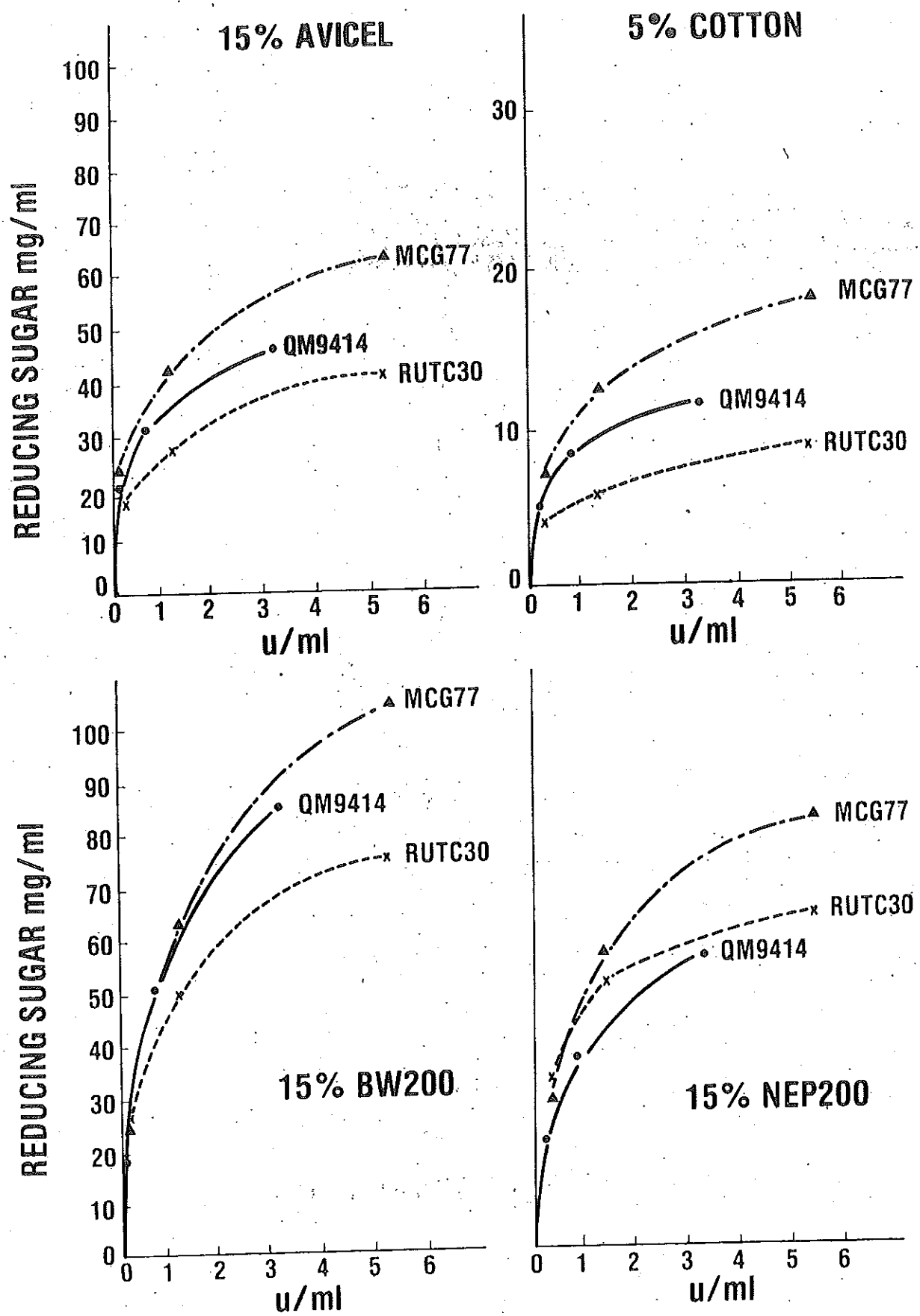

Figure 4. Saccharification of cellulose substrates by T. reesei mutants.

(Bissett et al. 1980)

TASK II SUBSTRATE AND SUBSTRATE PRETREATMENT

OBJECTIVES

To evaluate the effectiveness of various pretreatment processes on the susceptibility of waste cellulosic substrates to enzymatic hydrolysis by T. reesei cellulase. The ultimate goal is to develop an inexpensive pretreatment process for incorporation into the U.S. Army Natick R&D Laboratories process for the enzymatic conversion of cellulosic wastes to glucose. In addition, ongoing effort is directed toward the evaluation of waste cellulosic substrates in order to discover as many as possible which are amenable to this process.

ACCOMPLISHMENTS

A. Poplar Initial Moisture Content and Compression Milling Efficiency

Moisture contents of poplar shavings samples were adjusted to a range of 6% to 40% followed by compression (two roll) milling for 15 passes at a 10 mil roll clearance. Pretreatment effectiveness was evaluated by measuring the enzymatic hydrolysis susceptibility of these samples.

The results (Figure 1) indicate that the compression milling is most efficient when the poplar shavings are fed to the mill at approximately 12% moisture. A sharp decline in milling effectiveness is noted when the feedstock moisture content is above or below the optimum value.

Previous data (Quarterly Report Nos. 13 and 15) have shown there exist optimum moisture contents with respect to compression milling of corn stover, newspaper, urban waste and eastern spruce and in each instance the optimum

was unique. With the addition of this poplar data, we have shown that optimum moisture contents exist for at least one representative from the three primary lignocellulosic classes; i.e., hardwoods, softwoods and agricultural residues.

B. High Pressure Roll Milling

Development of a cost effective continuous compression mill pretreatment process depends in large part on minimizing the number of feedstock passes between the rolls. Previous studies (Quarterly Report No. 2) showed that as the roll clearance was decreased to an equipment limited 10 mils, (unloaded), roll milling efficiency improved. The effect of increasing the compressive force on the feedstock by narrowing the roll clearance to less than 10 mils was examined in an effort to further reduce the number of passes required for effective pretreatment.

A 4-high metals cold rolling mill as represented in Figure 2 was used. Since we do not have equipment of this type, engineering trials were conducted using the Farrel mill located at the General Electric Corporate R&D Center in Schenectady, NY. This equipment has two even speed, vertically stacked working rolls (6.5" dia. x 15") supported by two 21" dia. x 15" back-up rolls. Working roll speeds were variable up to 160 feet/min. To process cellulosic materials, the mill was modified by adding a ram feeder, collection bin, and knife scrapers on both working rolls. A recording ammeter and digital voltmeter were connected to each working roll D.C. motor to measure energy input during compression milling.

Excellent results were obtained with a pure cellulose. Enzymatic hydrolysis susceptibility of air dry absorbent cotton increased linearly

with the number of passes up to the three passes tested where an 8-fold improvement was observed over the untreated control (Figure 3).

When newspaper (air dry) was processed through the mill, the number of passes required to achieve improved susceptibility decreased as the roll clearance was reduced (Figure 4). With only six passes at a 0 mil roll clearance enzymatic hydrolysis reducing sugar production was increased from 14 mg/ml to 24 mg/ml. More than 60 passes on our equipment set at an unloaded 10 mil roll clearance would be required to achieve similar results.

Since the G.E. owned equipment was available for only two runs, minimal data could be collected on the effects of feedstock moisture content on high pressure roll milling effectiveness. However, from the one experiment performed, the results were very encouraging (Figure 4). At a roll clearance of 0 mils, newspaper with approximately 24% initial moisture content was in three passes slightly more susceptible to enzymatic hydrolysis than the air dry newspaper treated for six passes at the same roll clearance.

A test was conducted to determine the effects of roll speed on the pretreatment of air dry newspaper (Table 1). At a fixed roll clearance of 0 mils, there was no apparent difference in enzymatic hydrolysis reducing sugar production over a range of roll speeds from 30 feet/min. to 110 feet/min. These results are promising from a capital cost standpoint since higher roll speeds represent greater throughput of material for a given equipment size.

Energy input was the lowest obtained to date of any compression mill tested (Table 2) while the number of passes required was reduced to six or less. In the best case, a combination of proper moisture content (24%) and high pressure (0 mil roll gap) resulted in a 50% reduction in energy input as compared to air dry newspaper at 10 mils. Differences in equipment idling

characteristics account for some of this energy reduction. That is, the G.E. mill idles more efficiently than our rubber mills since the former has roller bearings and direct drive.

It is also significant that even at roll speeds of 110 feet/min., the G.E. mill energy consumption was only 0.20 kwh/lb. for the moist newspaper processed for three passes.

PLANS, NEXT QUARTER

1. Examine the effects of roll speed ratio on the moist compression milling of newspaper.

2. Initiate studies to recover hemicellulose sugars and/or lignin prior to compression milling.

3. Begin engineering trials on a modified production two roll mill (22" x 60") to determine processing capacities, energy input, and other parameters. Even speed rolls set as close together as possible will be used.

TABLE 1. EFFECT OF COMPRESSION MILL[1] ROLL SPEED ON NEWSPAPER ENZYMATIC HYDROLYSIS[2]

NO. OF PASSES	ROLL SPEEDS (FPM)	24 HR. REDUCING SUGARS (MG/ML)
1	30	17.0
	70	18.0
	110	19.8
3	30	17.5
	70	22.8
	110	22.0
	160	20.0
6	30	24.2
	70	25.2
	110	25.0

[1] FARREL 4-HIGH METALS ROLLING MILL, 6½" x 15" WORK ROLLS, δ = 0 MILS

Table 2. Effect of Mill Type on Compression Milling Efficiency in the Pretreatment of Newspaper

Mill Type	Newspaper Moisture Content (%)	Roll Clearance (mils)	No. of Passes	Roll Speeds (f.p.m.)	Enz. Hydrolysis[1] 24 Hr. Reducing Sugars (mg/ml)	Specific Energy Input (kwh/lb.)
Farrel Rubber Mill (10" x 20")	6	10	60	53/48	24.0	0.40
	24	10	15	53/48	25.0	0.27
Farrel Metals Cold Rolling Mill (6.5" x 15")	6	0	6	30/30	24.0	0.21
	24	0	3	110/110	24.8	0.20

[1]T. reesei (QM9414) cellulase, 19 IU/gm substrate, 5% slurries, pH 4.8, 50°C.

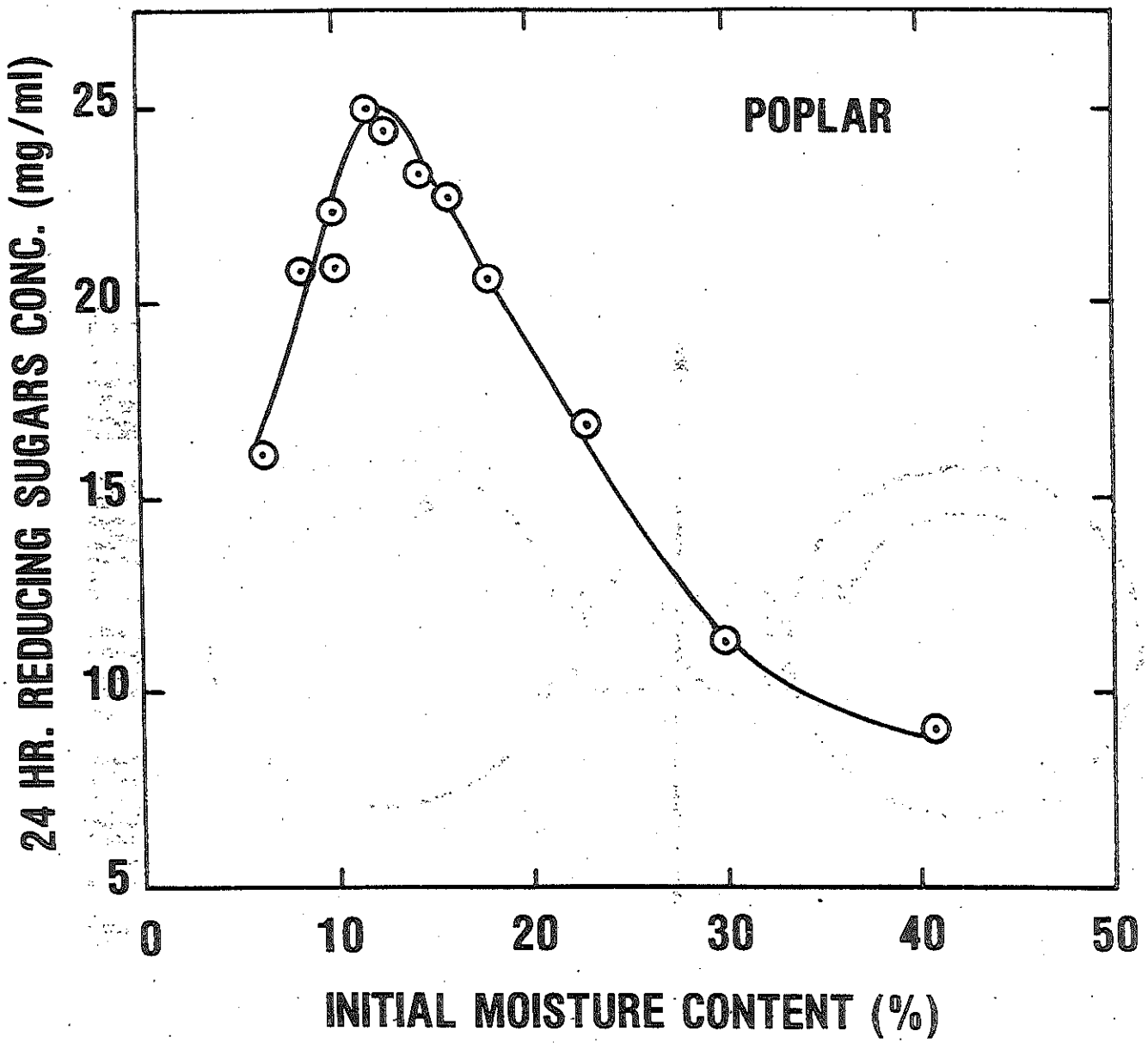

Figure 1. Effect of Poplar Initial Moisture Content on Compression Milling Pretreatment Effectiveness. Reliable two roll mill (6" x 13"), 15 passes, δ = 10 mils. Hydrolysis Conditions: 5% slurries, 19 FPU/gm substrate, pH 4.8, 50°C.

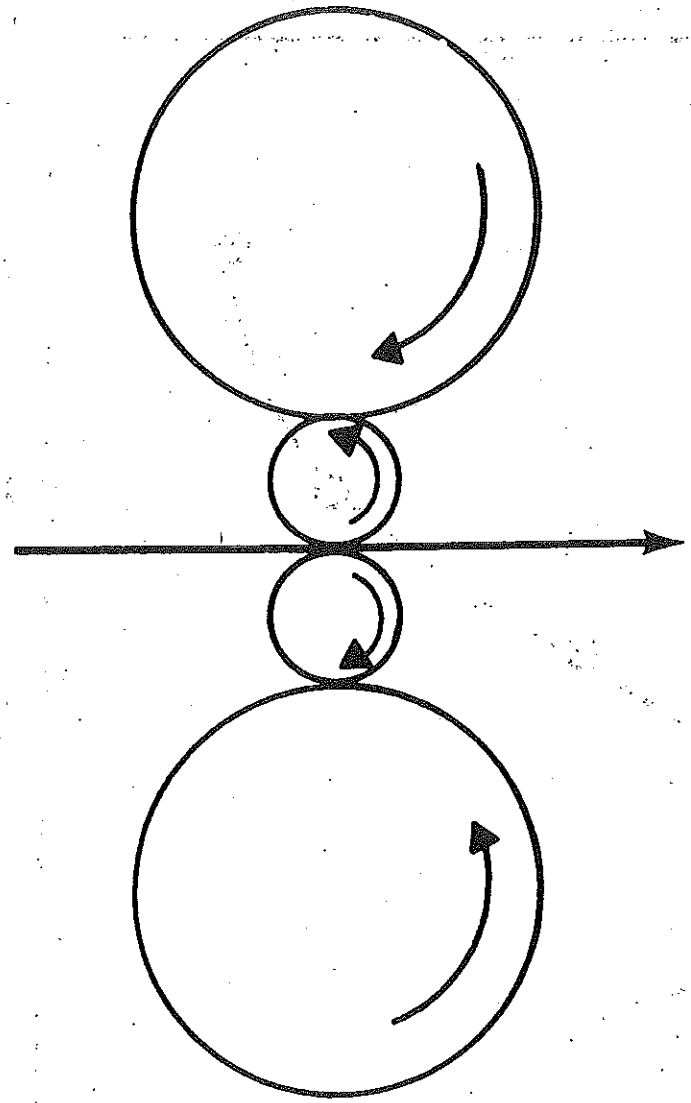

FIGURE 2. 4 HIGH ROLLING MILL

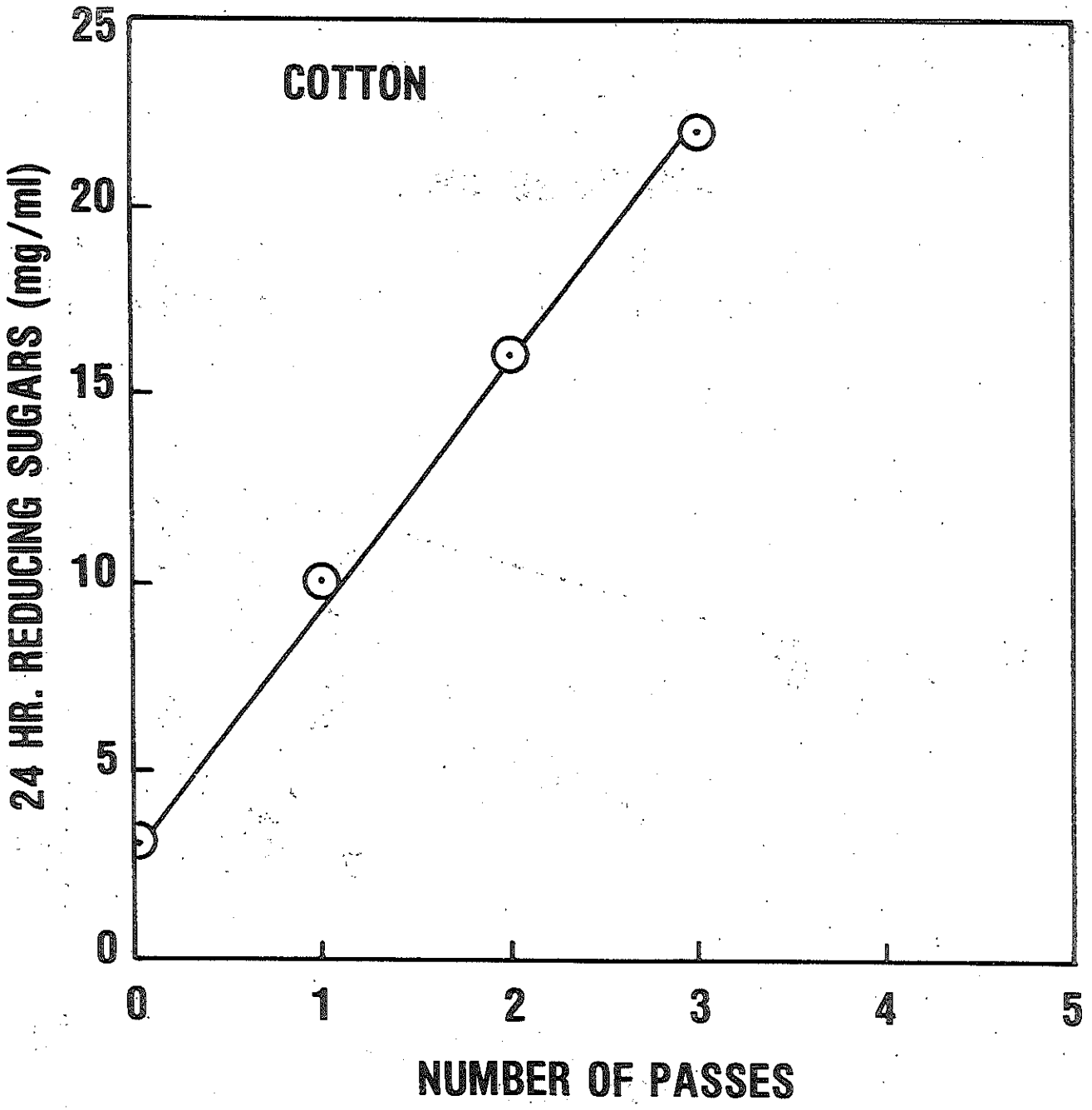

Figure 3. Relationship of Passes Through High Pressure Rolls and the Enzymatic Hydrolysis Susceptibility of Absorbent Cotton. Compression mill: Farrel 4-high metals cold rolling mill (6.5" x 15"), δ = 0 mils, $V_1 = V_2$ = 30 f.p.m. Hydrolysis Conditions: same as Figure 1.

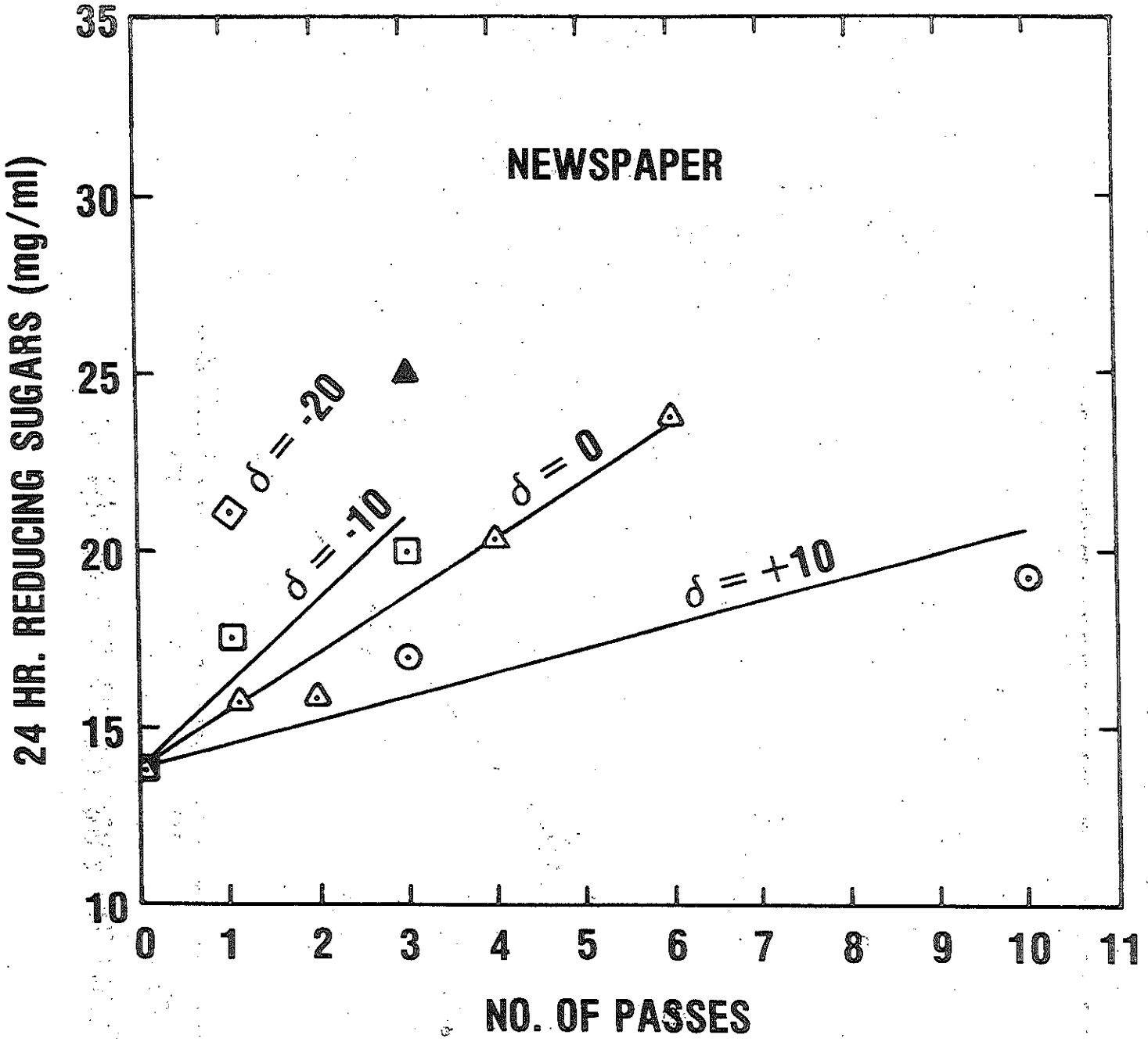

Figure 4. Effect of Compression Mill Roll Passes at Various Roll Clearances on Newspaper Enzymatic Hydrolysis Susceptibility. (◇), (△), (□): Farrel 4-high metals cold rolling mill (6.5" x 15"), $V_1 = V_2 = 30$ f.p.m., air dry newspaper; (▲): same as (△) except newspaper at 24% initial moisture content; (○): Farrel two roll mill (10" x 20"), $V_1 = 48$ f.p.m., $V_2 = 53$ f.p.m., air dry newspaper. Hydrolysis Conditions: same as Figure 1.

TASK III ENZYME PRODUCTION (PREPILOT SCALE)

OBJECTIVES

The principal objective of this task remains as the production of consistently high quality cell free cellulase with the development of a suitable technology for a commercial process as our ultimate goal.

ACCOMPLISHMENTS

It has been recognized that supplementation of Trichoderma cellulase with β-glucosidase enhances saccharification of cellulose. To accomplish this, development of a separate fermentation using Aspergillus phoenicis for the production of β-glucosidase looks promising.

During this quarter growth experiments were performed with Aspergillus phoenicis QM329 both in shake flasks and the 30 liter fermenter. In the shake flask experiments, six 300 ml shake flasks, 100 ml working volume buffered with .05 M citrate were each inoculated with 10 ml of 15 to 20 hour old suspended mycelia from a 2.8 liter Fernbach flask. Each flask was harvested and both dry weight and glucose levels were determined. The range of temperatures and pH examined were 25° - 45°C and 2.5 - 4.5 respectively. pH, in the range examined, had little effect on growth, whereas the optimum temperature was around 35°C. Mycelial pellet formation greatly affected growth, however the use of dimpled shake flasks minimized pellet formation. In shake flask cultures pellet formation was more evident at 40°C and 45°C than at the lower temperatures. Pellet formation was unavoidable in the 30 liter fermenter except when citrate (.05 M) was added in lieu of pH control (1.N NaOH).

The media for all experiments has the following: $(NH)_2SO_4$ - 2.1 g/1, KH_2PO_4 - 2.0 g/1, $MgSO_4 \cdot 7H_2O$ - 0.3 g/1, $CaCl_2$ - 0.3 g/1, glucose 10.0 g/1. Except where noted the media contained 0.5 M citric acid. The results of the experiment are shown in the following table. The data indicate that maximum growth 0.4 $hr.^{-1}$ occurs at about 35°C, remaining relatively constant between pH 3 to 4.5.

The growth rates for the Fernbach cultures were consistently lower than for the shake flask. Since the Fernbach cultures were older (15 to 20 hours) oxygen may be limiting its growth. Also significant is the low growth rates in the 30 liter vessel where citrate was absent. Pellet formation in these fermentations is probably responsible for the low growth. When citrate was present in the 30 liter media, the expected specific growth rate of 0.4 $hr.^{-1}$ was achieved.

At 35° the biomass yields from carbon and nitrogen were 0.54 and 9.5 g/g. At 40° the yields were 0.49 and 11.1 g/g. These yield values were obtained at pH 4.5.

PLANS, NEXT QUARTER

Work will continue with A. phoenicis to develop fed batch and continuous fermentations for β-glucosidase production.

Specific Growth Rate of Aspergillus phoenicis QM329 on 1% Glucose

Temperature °C	pH						
	2.5	3.0	4.0	4.5	4.5[1]	4.5[2]	4.5[3]
25	0.20	0.21	0.26	0.26	0.20		
30	0.37	0.36	0.36	0.36			
35		0.38	0.38	0.39	0.31	0.15-0.28	0.40
40	0.36	0.37	0.33	0.37	0.31		
45					0.20		

1. 2.8 liter Fernbach
2. 30 liter fermenter without citrate
3. 30 liter fermenter with citrate

TASK IV SACCHARIFICATION (PREPILOT SCALE)

OBJECTIVES

The process by which cellulosic substrates are enzymatically hydrolyzed is affected by many factors. These include the type and condition of the substrate, its concentration, the activity of the enzyme used, the reaction pH, temperature, and the reaction time. The optimum for each of these must be defined economically - that is, the effect of variations in each reaction parameter on the cost of the process.

Utilization of chemical process equipment presents different problems in terms of reaction control, maintenance, auxiliary equipment, apparatus. Economic analysis for large-scale operation can only be accurately made from data gathered from pilot-scale equipment. Consequently, all process changes must be proven effective at the prepilot level.

ACCOMPLISHMENTS

During the past quarter a series of runs were made using an attritor mill as a hydrolysis vessel. This was an attempt to scale up the shake flask experiments that used glass beads in the slurry (Quarterly Report No. 15 dated 12 January 1980). Runs were made using both ball milled newspaper (NEP200) and hammer milled newspaper (HMNP) with various glass beads to slurry ratios. Table 1 shows a 5 and 24 hour comparison between shake flask and attritor mill runs. For NEP200 slightly more sugar is produced both in 5 and 24 hours in the attritor mill than in shake flasks except for the 7:3.5 slurry to bead ratio for 24 hours. This data shows that it is possible

to scale up from shake flasks to production type equipment when using glass beads. The attritor mill produces about 10% less sugar in 5 hours than shake flasks not utilizing glass beads produce in 24 hours. It takes between 1.08 and 2.85 kilowatt hours (kwh) of electrical energy to run the attritor mill for 5 hours with the lowest power consumption for the 7:1.75 slurry to bead ratio. It takes an additional 3.03 to 4.85 kwh to run for 24 hours and an additional 44 to 64% more sugar is produced. Process economics will dictate whether it is feasible to run as long as 24 hours.

The 5 and 24 hour yields for the HMNP were also higher in the attritor mill than they were for the shake flasks except for 10% HMNP at 24 hours. It is believed that this was due to a severe pH excursion to 8.8 sometime after the 5 hour mark of the run. The 24 hour 15% HMNP shake flask test without beads yielded 39.7 mg/ml of reducing sugars while with beads the yield was 40.0 mg/ml. It is believed that the relatively high concentration of HMNP prevented the beads from moving thereby reducing the mechanical action necessary to improve the yield.

Table 2 summarizes the results of shake flask experiments using three enzyme strains on 15% NEP200, both with and without beads. MCG77 produces more sugar than both QM9414 and C30 at all three enzyme strengths, with and without beads. The use of beads improved sugar yields by as little as 15% for 3.0 IU/ml of C30 to as much as 54% when using 1.0 IU/ml for MCG77.

Table 3 summarizes the results of a set of 24 hour shake flask experiments using C30 enzyme both with and without β-glucosidase in shaken and unshaken flasks. In all cases except one the shaken flasks produced more sugar than the unshaken, but in many instances the results were nearly the same. In

most cases the addition of β-glucosidase improves the results for the unshaken flasks more than for the shaken flasks. The fact that the unshaken flasks produced nearly as much sugar as the shaken flasks is not surprising as recently reported (Reference 1) that unshaken flasks using C30 enzyme on Avicel actually produced more sugar than the shaken flasks. This was especially true for long time periods (70 hours). This is attributed to the less stable cellobiohydrolase component of the enzyme. This data shows that in production equipment all we need is enough agitation to maintain temperature and pH control.

In the continuing effort to produce as much sugar in as short a period of time as possible, a pilot scale run using 25% NEP200 and 7.5 IU/g unfiltered C30 enzyme (E/S = 30 IU/g) with 3.8 IU/g β-glucosidase yielded 100 mg/ml of total sugar in 17 hours, 124 mg/ml in 50 hours, 135 mg/ml in 75 hours and 138 mg/ml in 100 hours. The best run in terms of total sugar production used 30% NEP200 and 6.9 IU/g C30 with 3.6 IU/g β-glucosidase produced 105 mg/ml of sugar in 5 hours, 152 mg/ml in 50 hours and 160 mg/ml in 100 hours. Comparing these runs the value of high substrate concentration can be seen for producing high sugar yields.

PLANS, NEXT QUARTER

1. Additional hydrolysis runs in attritor mill will be performed.
2. Additional enzyme strains will be tested on NEP200 in shaken and unshaken flasks.
3. Pilot scale runs using wet two roll milled newspaper and MCG77 enzyme will be performed.

4. Work will continue using unfiltered enzyme for pilot scale runs.

REFERENCES

1. Reese, E. T.; and Mandels, M. 1980. Stability of Cellulase of T. reesei Under Use Conditions. Biotech. Bioeng. Vol. XXII. p. 323-335.

Table 1. Summary of Attritor Mill Hydrolysis Experiments using Different Bead to Slurry Ratios and a Comparison with Similar Shake Flask Experiments.

ENZYME	SUBSTRATE	SHAKE FLASK REDUCING SUGARS				ATTRITOR MILL REDUCING SUGAR mg/ml SLURRY TO BEAD RATIO							
		No Beads		Beads		7:7		7:3.5		7:1.75		10:0	
		5 Hrs	24 Hrs	5 Hrs	24 Hrs	5 Hrs	24 Hrs	5 Hrs	24 Hrs	5 Hrs	24 Hrs	5 Hrs	24 Hrs
2.0 IU/g C30 1.15 IU/g β-glucosidase	15% NEP200	32.2	49.7	40.8	66.4	45.8 (2.85)	67.5 (6.25)	44.5 (1.60)	64.1 (6.45)	41.3 (1.07)	68.0 (4.10)	32.1 (1.08)	54.5 (4.59)
2.0 IU/g C30 1.15 IU/g β-glucosidase	15% HMNP	25.6	39.7		40.0	DNR	DNR	38.7 (2.61)	41.1 (6.8)	Beads Added 18.2 (0)	36.0 (5.6)	28.9 (2.2)	47.2 (12.2)
2.0 IU/g C30 1.15 IU/g β-glucosidase	10% HMNP		24.0		40.2	DNR	DNR	26.4 (2.09)	28.3 (7.37)	DNR	DNR	DNR	DNR
1.0 IU/g C30 0.57 IU/g β-glucosidase	5% HMNP		12.3		19.3	DNR	DNR	15.6 (1.33)	23.7 (5.40)	DNR	DNR	DNR	DNR

DNR - Did not run
Figures in parentheses represent kilowatt hours used

Table 2. Comparison of Sugar Yields from QM9414, MCG77 and C30 on 15% NEP200 with and without Beads at 400 RPM.

ENZYME	YIELD (mg/ml) No Beads	YIELD (mg/ml) Beads	PERCENT IMPROVEMENT
1.0 IU/ml C30	33.0	42.5	28%
1.0 IU/ml QM9414	31.6	47.8	51%
1.0 IU/ml MCG77	33.5	51.6	54%
2.0 IU/ml C30	42.9	60.1	40%
2.0 IU/ml QM9414	44.8	59.6	33%
2.0 IU/ml MCG77	49.0	64.7	32%
3.0 IU/ml C30	49.8	69.1	15%
3.0 IU/ml QM9414	52.6	69.1	31%
3.0 IU/ml MCG77	54.9	76.9	40%

Table 3. Sugar Yields from Experiments using NEP200 and HMNP Run in Shaken and Unshaken Flasks for 24 Hours at pH 4.8.

SUBSTRATE AND CONCENTRATION	ENZYME STRENGTH (IU/g)		SUGAR CONCENTRATION (mg/ml)	
	C-30	β-glucosidase	Shaken	Unshaken
15% NEP200	2.0	None	43.7	36.8
15% NEP200	2.0	1.1	45.6	44.3
15% NEP200	2.0	2.0	52.8	46.0
15% NEP200	2.0	3.0	55.2	53.9
5% NEP200	1.0	None	14.4	12.4
15% HMNP	2.0	None	28.7	27.3
15% HMNP	2.0	1.1	29.6	29.1
15% HMNP	2.0	2.0	34.5	29.6
15% HMNP	2.0	3.0	38.6	38.9
5% HMNP	1.0	None	11.1	10.4
20% HMNP	2.5	1.4	41.7	37.7
25% HMNP	3.0	1.7	50.2	49.0

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